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Targeting Molecular Chaperones in Diabetic Peripheral Neuropathy

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1. Introduction

1.1 Diabetic peripheral neuropathy

Diabetes Mellitus (DM) is estimated to affect 347 million patients globally as of 2008 (Danaei *et al.*, 2011) and this number is expected to double by 2030 (Wild *et al.*, 2004). DM results from the failure of our body to generate and/or respond to insulin, the principal hormone regulating uptake of glucose from the blood stream, leading to abnormally high blood glucose levels, termed hyperglycemia. Sensory neurons absorb glucose as a direct function of extracellular glucose concentration instead of insulin-mediated glucose uptake and are particularly susceptible to the cumulative metabolic insults imposed by chronic hyperglycemia (Tomlinson and Gardiner, 2008). This damage leads to secondary microvascular complications that contribute to the progression of diabetic neuropathy. Diabetic peripheral neuropathy (DPN) is the most prevalent complication of DM (Tahrani *et al.*, 2010), also comprises the most common form of peripheral neuropathy globally, surpassing leprosy in this aspect (Harati, 2010; Martyn and Hughes, 1997). Depending on the case definitions used, 30-70% of patients with either type 1 or type 2 DM are diagnosed with some form of peripheral neuropathy (National Institutes of Diabetes and Digestive and Kidney Diseases). A distal symmetric sensorimotor polyneuropathy is the most frequent manifestation of DPN. Patients with this form of DPN are predisposed to foot ulceration and increased risk of amputation; ulcerative complications from DPN account for approximately 87% of non-traumatic lower extremity amputations. In addition to this traumatic medical event, DPN is also a major contributing factor to the development of joint deformities, limb threatening ischemia as well as other various neurological dysfunctions (Harati, 2010). This greatly reduces the quality of life in people with DM through increased disability and is assuming more hospitalizations than all other diabetic complications combined (Mahmood *et al.*, 2009). As a consequence, approximately $15 billion are spent on DPN annually in the US, causing a major drain on healthcare expenditure (Rathmann and Ward, 2003). With the staggering individual, social and economic burden brought about by DPN-associated morbidity and mortality, development of effective treatments that prevent and/or reverse DPN are needed but currently unmet.

1.2 Clinical features

DPN encompasses a wide spectrum of clinical and subclinical syndromes differing in their pattern of neurological involvement, anatomic distribution, specific neuropathic

alterations, risk covariates and course of development. As mentioned in the last section, distal symmetric sensorimotor polyneuropathy represents the most common type of involvement and affects 90% of patients with DPN (Harati, 2010). Damage to nerves initially begins with the longest axons, which accounts for the loss of sensation having a "stocking-glove" distribution. Degeneration continues to progress proximally in a dying-back, length-dependent pattern. (Boulton and Malik, 1998). Although post-mortem analysis indicates that all types and sizes of fibers are affected, sensory symptoms predominate over motor deficits at least in the early phase, likely due to the longer (and therefore more susceptible) axons needed to reach the epidermis. Patients diagnosed with this form of neuropathy often display symptoms such as paradoxical association of numbness with allodynia and dysesthesia associated with small fiber sensory dysfunction. As DPN advances, large sensory and motor fibers also become impaired which results in loss of vibratory sensation, proprioception, slowed nerve conduction velocities (NCV) and progressive fiber loss that culminates in irreversible neurodegeneration (Habib and Brannagan, 2010; Harati, 2010; Tahrani *et al.*, 2010).

1.3 Risk factors

Several landmark prospective studies enrolling a large cohort of type 1 diabetics, including the multicenter Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) observational study, have definitively established chronic hyperglycemia (mean glycosylated hemoglobin [HbA1c]> 6.5%) as a causative factor of DPN (Albers *et al.*, 2010; DCCT, 1988). This is strengthened by the fact that reduction in the incidence and progression of DPN can be achieved with rigorous glycemic control through intensified insulin therapy (DCCT, 1995; UK Prospective Diabetes Study Group, 1998). However, the fact that some patients still developed DPN even with good glucose control suggested the presence of other etiological factors. Indeed, duration of DM, age, obesity, hypertension, hyperlipidemia, cardiovascular disease, genetic predisposition and presence of other microvascular complications such as retinopathy and nephropathy also significantly increase an individual's susceptibility to DPN (Harati, 2010; Tesfaye and Selvarajah, 2009). Whether modifying some of these factors would help prevent or retard the development of DPN awaits further examination.

1.4 Pathogenesis

With years of ongoing efforts, a number of biochemical events have been identified as important mediators linking hyperglycemic stress to the development of DPN: increased oxidative stress, formation of advanced glycation end-products (AGEs), overflux of glucose through polyol and hexosamine pathways, abnormal activity of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NK-κB), neuroinflammation as well as impaired neurotrophic support. Unfortunately, none of the compounds developed against these targets has shown unequivocal effectiveness in preventing, reversing, or even slowing the neuropathic process in humans (Mahmood *et al.*, 2009; Obrosova, 2009; Tahrani *et al.*, 2010). A principal reason to explain these failures is the complexity of the pathogenesis of DPN since this array of molecular targets and pathways do not contribute to its pathophysiological progression in a temporally and/or biochemically uniform fashion. To date, the only effective strategy and gold standard method for preventing and treating DPN remains aggressive glycemic control, supplemented by FDA-approved medications providing pain relief. However, many patients struggle to maintain normoglycemia and

the eventual development of irreversible neurodegeneration has rendered our current management palliative at best. Clearly, the rational identification of new molecular paradigms that identify "druggable" targets is of high scientific impact to understanding the pathobiology of DPN and offers translational potential for improving its medical management. In this regard, emerging evidence has unraveled a fundamental but previously under-recognized alternative that modulating molecular chaperones, or heat shock proteins, affords a novel approach toward improving hyperglycemia as well as the function of myelinated and unmyelinated fibers in DPN. The remainder of this chapter will focus on a review of the functional, pathological and therapeutic implications of heat shock proteins in DM and DPN.

2. Scientific rationale for targeting molecular chaperones

2.1 Functions of heat shock proteins: "Molecular lifeguards"

Over 40 years ago, Ritossa observed that cells from the salivary gland of drosophila responded to an elevation in temperature with increased activation of certain sets of genes indicated by a "puffing" pattern of polytene chromosomes (Ritossa, 1962). A decade later, it was uncovered that the "puffs" were a result of a rapid and robust synthesis of a special group of proteins that are crucial for the protection and recovery from increased protein damage caused by heat stress, which would otherwise be lethal. These proteins were hence termed heat shock proteins (HSPs) and their induction was named the heat shock response (HSR). Abundant evidence has since accumulated suggesting that not only does this response exist in organisms ranging from prokaryotic bacteria to humans, but that it is also induced by a plethora of external and internal stimuli, including ischemic, hypoxic, chemical, inflammatory, oxidative and mechanical stress (Jaattela and Wissing, 1992). The universal nature of this phenomenon suggests that HSPs, or "stress proteins", play a critical role in cellular homeostasis. Indeed, blocking HSP induction mitigated the protective effect of mild heat-shock against subsequent death-inducing insults in cells (Park *et al.*, 2001). Most if not all HSPs function as "molecular chaperones", which provide a first line of defense against misfolded and/or aggregated proteins. By utilizing energy derived from ATP hydrolysis, molecular chaperones: 1) assist in the correct folding of nascent proteins during *de novo* synthesis or refolding of damaged proteins; 2) transport newly synthesized proteins to target organelles; 3) help maintain the correct tertiary structure of the protein, prevent and solubilize abnormal protein aggregates and 4) target severely damaged and irreparable proteins to proteasomal or lysosomal disposal. Apart from their "housekeeping" chaperoning function, increasing evidence suggests that HSPs are involved in other cellular processes such as inflammation and apoptosis. Transcription of HSPs during the HSR is regulated by heat shock factors (HSFs), of which HSF-1 is the prototype and master activator in vertebrates. Its importance to the HSR is underscored by the fact that genetic deletion of HSF-1 prevents HSP gene transactivation and development of thermotolerance as well as a loss of protection against inflammation (McMillan *et al.*, 1998; Pirkkala *et al.*, 2000; Xiao *et al.*, 1999; Zhang *et al.*, 2002). HSF-1 is largely repressed and kept as an inert monomer in the cytosol by the chaperone HSP90. This interaction is disrupted by certain types of cell stress and leads to the dissociation of HSF-1. Following homotrimerization, phosphorylation and translocation to the nucleus, HSF-1 activates transcription of HSPs by binding to highly conserved sequences known as heat shock elements (HSEs) within the promoter region of multiple heat-shock genes. However, the exact phoshorylation sites through which HSF-1 is

activated is not settled (DeFranco *et al.*, 2004). Although different classifications can be ascribed based on function, cellular localization and expression pattern, HSPs are widely categorized according to their molecular sizes into five families, comprising HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs with a molecular mass less than 40 kilo-daltons (kDa) (Feder and Hofmann, 1999). Specific HSPs that are particularly implicated in the context of DM and DPN are briefly discussed below.

2.1.1 HSP90: Seeking effective pharmacological "heat-shock therapy"

HSP90 is one of the most prevalent cellular proteins as it accounts for 1-2% of total protein and is essential for the cell survival in most eukaryotes; the latter is clearly indicated by the embryonic lethality of HSP90 knockout mice (Yeyati and van Heyningen, 2008). HSP90 is ubiquitously expressed in the cytosol where it is responsible for post-translational maturation of a variety of nascent polypeptides as well as solubilization and refolding of misfolded and damaged proteins after stress. The most predominant isoforms are stress-inducible HSP90α and constitutive HSP90β that primarily localize in the cytoplasm. Other homologues include Grp94 in the endoplasmic reticulum and the mitochondria matrix protein HSP75/TRAP-1. All HSP90s are highly conserved evolutionarily in their structures and share an N-terminal ATPase domain, a connective linker region, a middle domain involved in binding substrates (client proteins). HSP90 also has a C-terminal domain that is responsible for interactions with various partner proteins and co-chaperones which provide a coordinate regulation over its diversified functions (Peterson and Blagg, 2009; Soti *et al.*, 2002). Because numerous HSP90 client proteins are involved in cell growth, differentiation, and survival secretion, inhibitors directed against the HSP90 N-terminal ATP binding domain induce simultaneous degradation of a wide variety of client proteins and are potent chemotherapeutic agents in cancer (Peterson and Blagg, 2009; Soti *et al.*, 2005). An important aspect of N-terminal HSP90 inhibitors in treating malignant phenotypes is that the drugs preferentially inhibit HSP90 and induce client protein degradation in malignant versus normal cells (Luo *et al.*, 2008). This selectivity may be due to the upregulation of HSP90 to accommodate the dependency of malignant cells on overexpressed oncogenic client proteins (Chiosis *et al.*, 2003) and/or an increased affinity of N-terminal inhibitors for HSP90-oncoprotein complexes in cancer cells (Kamal *et al.*, 2003). Although this selectivity aids the clinical efficacy of N-terminal HSP90 inhibitors, enthusiasm for their use has been hampered because induction of client protein degradation and cytotoxicity occurs at drug concentrations that also activate an antagonistic aspect of HSP90 biology, the promotion of the cytoprotective HSR.

Since N-terminal HSP90 inhibitors promote the HSR and decrease protein aggregation, they also have possible utility in treating neurodegenerative diseases associated with protein misfolding. In this regard, N-terminal HSP90 inhibitors decrease tau protein aggregation in Alzheimer's disease models (Dickey *et al.*, 2007; Luo *et al.*, 2007) and improve motor function in spinal and bulbar muscular atrophy (Waza *et al.*, 2005). Although a similar selectivity exists for the use of N-terminal inhibitors in treating neurodegenerative diseases (Dickey *et al.*, 2007), this selectivity does not circumvent the issue related to dissociating client protein degradation from induction of the HSR. Now, the inverse caveat exists; despite being neuroprotective, induction of client protein degradation may produce cytotoxicity. Thus, developing a highly effective HSP90 inhibitor for treating neurodegeneration requires establishing a sufficient therapeutic window that avoids increased client protein

degradation that may antagonize a cytoprotective HSR. HSP90 also contains a C-terminus ATP binding domain that weakly binds the antibiotic novobiocin. Similar to N-terminal inhibitors, novobiocin can promote client protein degradation and induce a HSR. Through systematic modification of the coumarin ring pharmacophore of novobiocin, KU-32 was identified as a lead compound that exhibits at least a 500-fold divergence of client protein degradation from induction of HSP70 (Urban *et al.*, 2010). This divergence provides an excellent therapeutic window to promote neuroprotection in the absence of toxicity. Thus, non-selective uptake and off-target toxicity is not a confounding issue as discussed above. In support of this safety, administering 400 mg/kg of KU-32 to mice (20x > our typical dose) did not induce overt toxicity or histopathological changes on all organs examined. In our study with STZ-diabetic mice, treatment of mice with KU-32 effectively reversed the development of sensory hypoalgesia in an HSP70-dependent manner (Urban *et al.*, 2010). This study provides encouraging evidence that inhibition, or modulation of HSP90 offers a potent "heat-shock therapy" in treating DPN.

2.1.2 HSP70: Potent "cytoprotectant"

The 70-kDa HSPs are found in many of the major subcellular compartments: cytosolic heat shock cognate protein 70 (HSC70) with a molecular weight of 73kDa, the stress-inducible cytosolic HSP70 (HSP72 in humans), the endoplasmic-reticulum (ER)-localized glucose-regulated protein 78 (Grp78/BiP) and the mitochondrial glucose-regulated protein Grp75/mortalin. Although sharing 80% homology with HSP70, HSC70 is constitutively expressed and only moderately inducible whereas expression of HSP70, Grp78 and Grp75 can be induced by stressful stimuli to varying degrees, with HSP70 being the most robust. After cell stress, HSP70 appears in both the cytoplasm and nucleus and helps repair damaged proteins. Similarly, induction of Grp78/BiP and Grp75 are essential for maintenance of ER and mitochondrial proteostasis, respectively (Richter-Landsberg and Goldbaum, 2003). Chaperones rarely work alone, and usually associate with each other and/or other co-factors to carry out distinct functions. For example, binding of HSP40 co-chaperone to HSP70 often facilitates substrate folding and refolding (Michels *et al.*, 1997; Minami *et al.*, 1996) whereas association of HSP70 with HSP90 via HSP-organizing protein (HOP) typically targets proteins towards proteasomal degradation (Sajjad *et al.*, 2010). Due to the importance of HSP70 in the maintenance of cellular homeostasis and defense against various insults, its deficiency or dysfunction has been linked to numerous human diseases (Muchowski and Wacker, 2005). However, direct targeting of HSP70 as a pharmacological objective has been complicated by its high conservation and ubiquitous expression patterns. Nevertheless, there appear to be some compounds such as geranylgeranyl acetone (GGA) that directly modulate HSP70. GGA is currently the leading antiulcer drug in Japan (Ushijima *et al.*, 2005) and has been shown to be neuroprotective against cerebral ischemia, polyQ toxicity (Sajjad *et al.*, 2010) and recently in a diabetic monkey model to attenuate insulin resistance (Kavanagh *et al.*, 2011) via the induction of HSP70. As many of the other HSR inducers or co-inducers, the HSP induction mechanism of GGA is not elucidated but has been suggested via dissociation of HSF-1 from HSP70 by competitively interacting with HSP70 C-terminal peptide-binding domain, which is the HSF-1 binding sites during HSP70-HSF-1 negative feedback (Otaka *et al.*, 2007). If GGA indeed acts through this mechanism, a combination of GGA with HSP90 inhibitors might exert a robust "heat-shock paradigm".

2.2 HSP modulates insulin resistance

Skeletal muscle, heart, liver and adipocytes comprise about two-thirds of the cells in the body and depend on insulin for glucose uptake. Reduced or loss of response of these tissues to insulin, namely insulin resistance, is a key pathogenetic feature that leads to the development of frank type 2 DM and its complications, such as DPN. Since type 2 DM is accountable for more than 90% of all cases of DM worldwide (World Health Organization, 2011), inhibiting insulin resistance assumes an important role in managing DPN. Although the exact mechanisms leading to insulin resistance are yet to be elucidated, it has become apparent that excess free fatty acids, inflammatory cytokines, mitochondrial impairment and oxidative stress may activate multiple signaling pathways that negatively affect insulin signaling through inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1). In obesity for instance, induction of protein kinase C (PKC)-ε/θ through increased synthesis of diacylglycerol (DAG) from fatty acids has been linked to impaired insulin activity in skeletal muscle and liver (Schmitz-Peiffer, 2002; Schmitz-Peiffer *et al.*, 1997), presumably through activation of serine-threonine kinases including inhibitor of nuclear factor κB kinase-β (IKK-β) (Itani *et al.*, 2002; Shoelson *et al.*, 2003) and/or c-jun N-terminal kinase (JNK) (Hirosumi *et al.*, 2002) (Fig.1.). Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) secreted from hypertrophied adipose tissue and/or macrophages, can also stimulate JNK and IKK-β in various insulin-sensitive tissues and promote insulin resistance (Wellen and Hotamisligil, 2005). Particularly, phosphorylation of serine 312 (S312) in humans or S307 in rats by JNK and IKK-β renders this crucial signaling intermediate a poor substrate for the insulin-stimulated receptor (McCarty, 2006).

2.2.1 HSP inhibits inflammation and stress kinases

As natural inhibitors of above damaging events and their catalyzing enzymes, HSPs may provide a broad defense against insulin resistance. The first supporting evidence came from a small clinical study performed by Hooper over ten years ago. In this study, heat therapy on patients with type 2 DM through hot tub immersion six days a week for three consecutive weeks dropped their fasting plasma glucose and mean glycosylated hemoglobin levels (Hooper, 1999). Although the HSP levels were not measured in this trial, it documented an average rise of 0.8°C in body temperature, which is sufficient for HSP induction (Okada *et al.*, 2004). Despite the lack of proper controls and its exploratory nature, the credibility of this early report has been substantiated by similar studies from animal models of DM. For example, whereas high-fat diet evoked significant hyperglycemia, hyperinsulinemia, insulin insensitivity, glucose intolerance as well as JNK and IKK-β activation in skeletal muscles in rodents, weekly heat therapy upregulated HSP70 which paralleled an apparent attenuation of these metabolic characteristics (Chung *et al.*, 2008; Gupte *et al.*, 2009b). Chung *et al* further demonstrated that targeted overexpression of HSP70 in mouse skeletal muscles mimicked the effects of heat therapy in preventing fat-induced JNK phosphorylation, insulin resistance and fat deposition, suggesting that HSP70 might mediate the beneficial effects afforded by heat therapy (Chung *et al.*, 2008). Indeed, pharmacological induction of HSP70 with the hydroxylamine derivative BGP-15, α-lipoic acid or GGA improved glucose tolerance in insulin-resistant mice (Chung *et al.*, 2008), rats (Gupte *et al.*, 2009a) and monkeys (Kavanagh *et al.*, 2011), respectively. Notably, administration of a HSP co-inducer BRX-220 to STZ-diabetic rats dose-dependently reduced insulin resistance and was associated with improved peripheral sensory and motor nerve deficits (Kurthy *et al.*, 2002).

In Gupte's study of rats fed with high-fat diet, addition of an HSP70 inhibitor diminished the heat-induced JNK depression, indicating that inhibition of JNK activation by heat-shock is largely HSP70-dependent (Gupte *et al.*, 2009b). In fact, a role for HSP70 as a JNK inhibitor has been well-established *in vitro* and appears to be of physiological significance. For example, heat-shock preconditioning suppressed JNK activity and apoptosis in fibroblasts upon ultra violet exposure. However, this effect was mitigated when cells were transfected with antisense HSP70 oligonucleotides (Park *et al.*, 2001). The precise mechanism by which HSP70 inhibits JNK is at dispute. HSP70 may directly bind to JNK and inhibit its activation by upstream activating kinases and preventing phosphorylation of its substrate, c-jun (Geiger and Gupte, 2011; Park *et al.*, 2001). On the other hand, others have failed to detect an HSP70-JNK physical interaction through immunoprecipitation (Chung *et al.*, 2008). This disparity is confounding and awaits further scrutiny. In addition to a direct modulation on JNK itself, other reports also suggest that HSP70 may regulate JNK activity via effects on upstream kinases (Daviau *et al.*, 2006) and/or phosphatases (Meriin *et al.*, 1999).

2.2.2 HSP enhances mitochondrial function

Although the exact role of mitochondrial dysfunction in the development of insulin resistance is controversial, mitochondrial dysfunction is frequently found in insulin-resistant human or animals (Bonnard *et al.*, 2008; Morino *et al.*, 2006; Petersen *et al.*, 2004). It is believed that with obesity and nutrient overload, there is excessive beta-oxidation of fatty acids that is unmatched with a concomitant elevation in downstream mitochondrial enzyme activities and ATP demand. This uncoupled mitochondrial flux produces a buildup of beta-oxidation intermediates which promote proton leak and increased reactive oxygen species (ROS) generation, which in turn stimulates the aforementioned stress kinases that contribute to insulin resistance (Koves *et al.*, 2008). One way in which insulin resistance is alleviated by heat therapy might be through enhancing mitochondrial energetic flux and oxidative capacity. This is supported by the observation that heat treatment maintained citrate synthase and cytochrome oxidase activity, which were decreased with fat feeding. Likewise, a single heat treatment prevented impaired mitochondrial oxygen consumption and fatty acid oxidation in L6 muscle cells treated with TNF-α and palmitate, respectively (Gupte *et al.*, 2009b). These results are consistent with previous studies demonstrating that heat shock protected mitochondrial function against oxidative and ischemic injury (Polla *et al.*, 1996; Sammut *et al.*, 2001). Independent of heat treatment, transgenic mice overexpressing HSP70 in skeletal muscles possess higher citrate synthase and β-hydroxyacyl-CoA-dehydrogenase activity and are refrained from insulin resistance (Chung *et al.*, 2008). HSP70 plays an important role in mitochondria proteostasis since it facilitates import of nuclear encoded proteins into mitochondria via interaction with the mitochondrial protein import receptor Tom70 (Young *et al.*, 2003). In addition, overexpression of HSP70 decreased ROS formation and maintained mitochondrial respiration in glucose-deprived astrocytes (Ouyang *et al.*, 2006). Clinically, poor HSP70 mRNA expression is found in diabetic humans and correlates with reduced mitochondrial enzyme activity (Bruce *et al.*, 2003), increased JNK activity (Chung *et al.*, 2008) and the degree of insulin resistance (Kurucz *et al.*, 2002). Evidence from animal models of DM also supports this finding at the protein level (Atalay *et al.*, 2004; Kavanagh *et al.*, 2011). Given the profound impact of HSP on stress kinases and mitochondrial integrity, it is conceivable that reduced HSP expression might have unfettered the deleterious inflammatory signaling and redox homeostasis which accelerate the development of insulin resistance.

Thus, increasing HSP could be a powerful tool in combating insulin resistance to help prevent diabetic complications such as DPN (see Fig.1.).

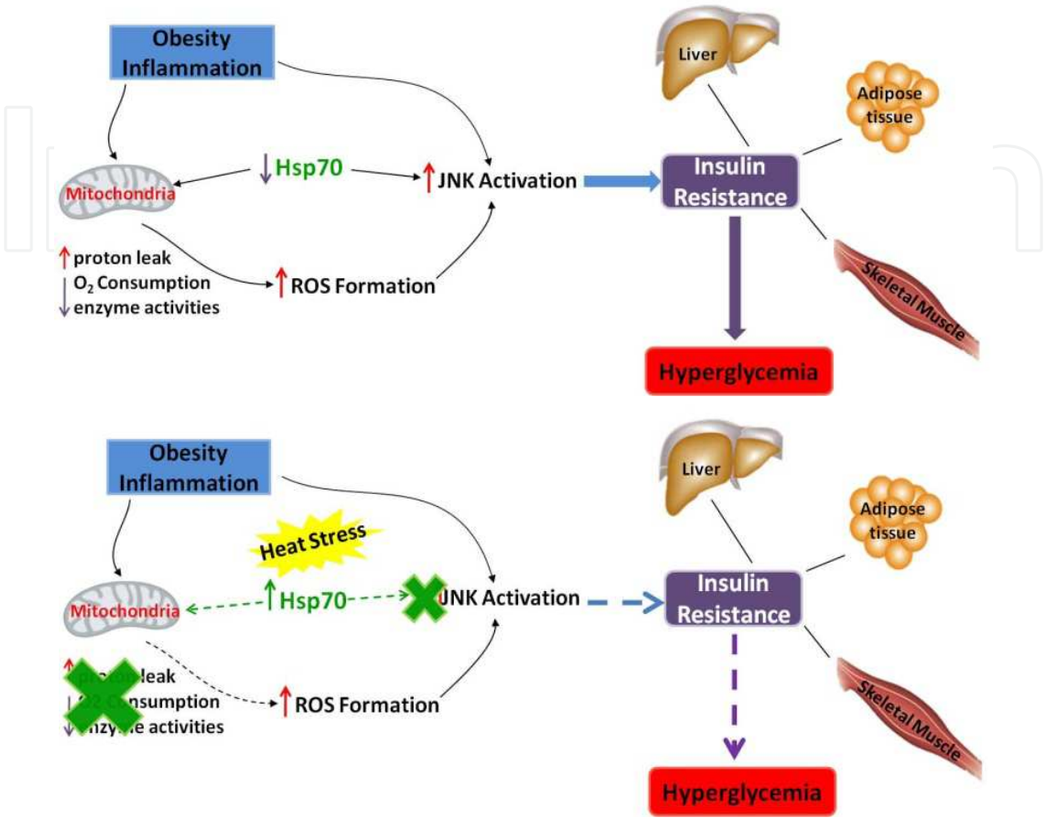

Fig. 1. Schematic of obesity and inflammation-induced insulin resistance and role for Hsp70 as an inhibitor of insulin resistance. Solid lines indicate stimulated pathways; dashed lines indicate inhibited pathways.

2.3 Impaired HSP Protection in DPN

Chronic hyperglycemia can trigger excess ischemic, hypoxic, oxidative and apoptotic stress. However, molecular chaperones, which normally respond by mounting a defense against such stresses, are often found paradoxically low in diabetic tissues. For example, in a couple of clinical studies enrolling type 2 diabetics and healthy controls, patients with DM had significantly lower mRNA levels of HSP70 and heme oxygenase-1 (HO-1, HSP32), a stress-inducible HSP that has antioxidant properties (Bruce *et al.*, 2003; Kurucz *et al.*, 2002). In a comparison of twins discordant for type 2 DM, muscle HSP70 levels are significantly lower in both diabetic twins and their non-diabetic but insulin-resistant monozygotic co-twins as compared to healthy controls. Particularly, the level of HSP70 mRNA inversely correlated with the severity of insulin-resistance and diabetic phenotype (Kurucz *et al.*, 2002). Chung *et al.* confirmed that there is a reduced expression of HSP70 protein in muscle from obese, insulin-resistant humans (Chung *et al.*, 2008). In type 1 diabetics diagnosed with polyneuropathy, a marked decreased in the HSP70 content in peripheral blood cells has also been reported (Strokov *et al.*, 2000). In contrast, this reduction was not observed in a recent

study of non-insulin-dependent diabetic males who were free of diabetic complications (Brinkmann *et al.*, 2011). This discrepancy may indicate a positive correlation between lower HSP70 expression and the presence of diabetic complications. In line with this hypothesis, physical or pharmacological therapies that improved neuropathic symptoms in type 1 and type 2 diabetics have demonstrated significant restoration of HSP levels (Hooper, 1999; Strokov *et al.*, 2000).

The association of decreased HSP70 expression with DM is also supported by studies in a variety of animal models. In diabetic monkeys, hepatic and plasma HSP70 was reduced by hyperglycemia in a dose-dependent fashion. In addition, long-term hyperglycemia also dose-dependently impaired the activation of liver HSF-1 in response to heat-shock, with a concomitant compensatory increase in total HSF-1 protein (Kavanagh *et al.*, 2011). In mice fed a high-fat diet, the induction of HSP70 by heat therapy was attenuated compared to chow-fed mice (Chung *et al.*, 2008). Evaluation of HSP levels in rats rendered diabetic with the β-cell toxin streptozotocin (STZ) has generated equivocal results: HSP70 and HSF-1 protein content was reported to be both decreased (Atalay *et al.*, 2004) and unchanged (Najemnikova *et al.*, 2007; Swiecki *et al.*, 2003) in heart and liver. The effects of DM on muscle and kidney HSP expression may be more cell-type specific since diabetic rats showed increased renal HSP70 and HSP25 immunoreactivity in the outer medulla but not in glomeruli (Barutta *et al.*, 2008). Species and genotype, duration and severity of DM, tissue vulnerability and antibody specificity/sensitivity may serve as contributing variables that underpin these differing results. Alternatively, these seemingly contradictory observations might actually reflect the heterogeneous nature and pathological progression of DM. This is supported by findings in dorsal root ganglia of spontaneous diabetic BB/Wor rats (which develop β-cell dysfunction and neuropathy similar to that seen in human type 1 DM). Whereas an elevated expression of HSP70 and HSP25 was seen following 4-months of DM, HSP70 expression was significantly blunted after 10-months of DM and correlated with decreased neurotrophic components and degeneration of myelinated and unmyelinated fibers, indicative of advanced polyneuropathy (Kamiya *et al.*, 2006; Kamiya *et al.*, 2005). Therefore, despite the non-linear pattern of changing in HSP expression, the data suggest a possible inverse relationship between the level of HSP and neuropathological change in DM. Consistent with this notion, compounds known to co-induce HSP were able to improve diabetic peripheral neuropathy (Kurthy *et al.*, 2002) and wound healing (Vigh *et al.*, 1997). Although the development of diabetic complications is associated with a progressive decline in the expression of HSPs, mice with a genetic knock-out of the inducible isoforms of HSP70 (HSP70.1 and 70.3) did not develop any more severe neurophysiological deficits than were observed in wild-type diabetic mice (Urban *et al.*, 2010). Thus, HSP70 is not essential to the pathophysiologic development of DPN and the lack of a more severe neuropathy in the diabetic HSP70 knockout mice may suggest a compensatory involvement of other HSPs such as HSC70. Nevertheless, a genetic assessment in patients with DM identified an HSP70 gene polymorphism in association with an increased severity of diabetic foot ulceration, need for amputation and greater duration of hospitalization (Mir *et al.*, 2009). Similar to that observed in spontaneous diabetic BB/Wor rats, short-term DM (30 days) enhanced HSP induction in response to heat treatment in STZ-injected rats versus control (Najemnikova *et al.*, 2007). However, prolonged (8 weeks) hyperglycemia significantly blunted the HSR stimulated by endurance exercise training or heat stress in the same model (Atalay *et al.*, 2004; Swiecki *et al.*, 2003). On the other hand, although a 15-min heat-shock upregulated HSP70 in diabetic rat heart, it failed to protect against

myocardial ischemia/reperfusion injury (Joyeux *et al.*, 1999). Therefore, DM may have impaired the cytoprotective function of chaperones and some of the enhanced expression of HSP might reflect an overwhelmed cellular stress defense or overall failure of chaperones to function properly. Although a systemic and in-depth characterization of expression and functional change of nerve HSPs throughout the course of DPN is needed, it is tempting to speculate that chronic metabolic stress in DM produces a disturbed endogenous defense system and results in widespread tissue vulnerability to various insults which contribute to diabetic complications such as DPN. Despite the ambiguity cited above, these results collectively support that modulating chaperone function can have potential benefit on the progression of DM and its complications.

2.4 Direct role for HSP in neuroprotection

A direct cytoprotective effect of HSPs in nerves has been extensively characterized in an array of neurodegenerative disease models. For instance, transgenic overexpression of HSP70, HSP40 or HSP27, either separately or in combination, protected neurons from mutant or misfolded protein-induced toxicity in cell culture and animal models of familial amyotrophic lateral sclerosis (Bruening *et al.*, 1999; Takeuchi *et al.*, 2002), Alzheimer's (Magrane *et al.*, 2004; Shimura *et al.*, 2004a), Parkinson's (Klucken *et al.*, 2004) and polyglutamine (polyQ) expansion diseases (Jana *et al.*, 2000; Wyttenbach *et al.*, 2002). A large part of this protection was attributed to the ability of HSPs to decrease toxic protein aggregates through refolding or targeted degradation (Cuervo *et al.*, 2004; Klucken *et al.*, 2004; Shimura *et al.*, 2004b). Apart from chaperone function, both heat-shock preconditioning and overexpression of HSP70 and HSP27 improved neuronal survival in mice following focal or global cerebral ischemia (Kelly and Yenari, 2002); this protection was linked to HSPs interfering with inflammatory and apoptotic signaling pathways.

In contrast to the extensive evidence characterizing the neuroprotective effects of HSPs in protein conformational and neurodegenerative disorders, scant data has been published with regard to their possible benefit in diabetic nerve. To date, most relevant evidence is largely correlative in nature and comes from indirect evaluation of pharmacological HSP co-inducers. In STZ-induced diabetic Wistar rats, two known HSP co-inducing compounds, bimoclomol (Biro *et al.*, 1997) and BRX-220 (Kurthy *et al.*, 2002), independently reversed the slowing of motor and sensory conduction deficits as well as the ischemic conduction failure in sciatic nerve. Substantially advancing these findings, studies in our laboratory also demonstrated a potent reversal of pre-existing mechanical and thermal hypoalgesia in addition to an improved motor and sensory NCV in STZ-mice administered a small molecule HSP90 inhibitor, KU-32 (Urban *et al.*, 2010). This protection occurred without an obvious improvement in serum glucose or insulin levels, indicating that it is likely to be a direct effect in nerves, Schwann cells (SCs) or endothelium. Importantly, HSP70 appeared to be a critical component in the mechanism of protection by KU-32 as this compound showed no sign of ameliorating any of the clinical indices of DPN that developed in diabetic HSP70 knockout mice (Urban *et al.*, 2010). These studies, however, do not provide mechanistic insight as to whether and how HSP70 elicits a protective effect in diabetic peripheral nerves. Part of the reason comes from the fact that unlike most other neurodegenerative disorders that have been the focus of chaperone therapy, the etiology of DPN is not associated with the accumulation of a specific misfolded or aggregated protein. However, the efficacy of KU-32 in reversing several clinically relevant physiologic indices of nerve dysfunction

strongly supports that damaged proteins contribute to DPN. Although DPN is primarily a metabolic disease that encompasses a panoply of physiological and biochemical disturbances, hyperglycemic stress can increase oxidative modification of amino acids (Akude *et al.*, 2009; Obrosova, 2009) that can impair protein folding, decrease refolding or induce protein aggregation (Muchowski and Wacker, 2005). Oxidative stress can also contribute to mitochondrial dysfunction (Chowdhury et al., 2011) and aggregation within sensory axons in DPN (Zherebitskaya *et al.*, 2009). In this regard, increasing chaperones in nerves may provide an excellent endogenous protein "quality control" defense by enhancing protein folding and/or refolding. Additionally, evidence is accumulating that HSPs are capable of intersecting with multiple other molecular targets that contribute to DPN.

2.4.1 JNK inhibition

JNK activation has been suggested to mediate neurotrophic factor deprivation-induced apoptosis in cultured sympathetic, motor or cerebral granule neurons (Eilers *et al.*, 1998; Maroney *et al.*, 1998; Watson *et al.*, 1998). As mentioned earlier, the inhibition of JNK-mediated inflammation and apoptosis by HSP70 is well-established in non-neuronal cells. This anti-apoptotic effect of HSP70 was further extended in sympathetic neurons where virally-directed (Bienemann *et al.*, 2008) or compound-induced expression (Salehi *et al.*, 2006) of HSP70 suppressed JNK activity and subsequent apoptosis. Although the contribution of neuronal apoptosis to DPN is controversial (Cheng and Zochodne, 2003), high concentrations of glucose induced apoptotic change in superior cervical gangalion neurons (Russell and Feldman, 1999). JNK inhibition might therefore partially contribute to nerve protection by HSP70 (see Fig.2.). In agreement, pretreatment of rat embryonic sensory neurons with KU-32 prevented glucose-induced cell death (Urban *et al.*, 2010). In amputated limbs from type 1 diabetic patients, sural nerve biopsies revealed a 2.5-fold increase in JNK activity (Purves *et al.*, 2001). Both hyperglycemically stressed adult sensory neurons (Purves *et al.*, 2001) and diabetic rat DRGs and sural nerve (Fernyhough *et al.*, 1999) underwent prolonged JNK activation that correlated with increased hyperphosphorylation of neurofilaments, the latter has been linked to axonal dystrophy seen in DPN (Fernyhough *et al.*, 1999). In addition, there is compelling evidence that the JNK pathway contributes to the pain sensitization of mechanical allodynia (Gao and Ji, 2008; Zhuang *et al.*, 2006), inflammatory hyperalgesia (Doya *et al.*, 2005) and diabetic neuropathic pain (Daulhac *et al.*, 2006). These observations support that JNK inhibition by HSP70 might help prevent development of distal axonopathy or neuropathic pain in DM.

2.4.2 Mitochondria protection

Investigation on mitochondrial dysfunction in diabetic nerves is a rapidly developing field. Sensory neurons from diabetic rats demonstrate significant decreases in mitochondrial respiration rate and complex I and IV enzyme activity, which is associated with impaired oxidative phosphorylation and diminished ROS formation (Akude *et al.*, 2011; Fernyhough *et al.*, 2010). Since HSP70 was closely linked to the maintenance and enhancement of mitochondrial function in diabetic muscle (Chung *et al.*, 2008; Gupte *et al.*, 2009b), it is reasonable to test the hypothesis that HSP70 might help preserve the competency of mitochondrial bioenergetics in neurons under glucose-driven metabolic stress. In support of this, over-expression of HSP70 increased the activity of complexes III and IV of the respiratory chain from hypoxia/reoxygenation injury in heart (Williamson *et al.*, 2008).

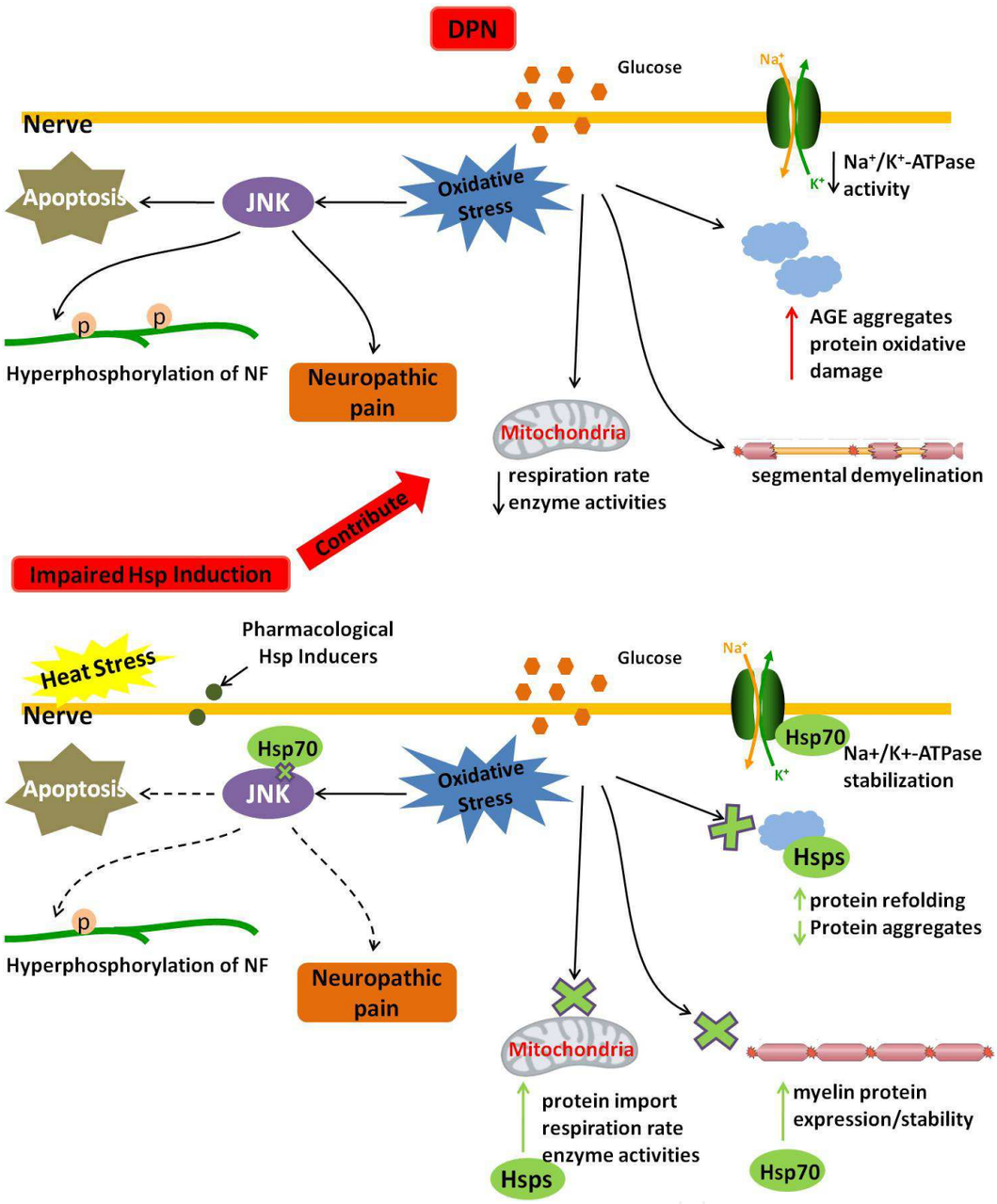

Fig. 2. Potential mechanisms underlying Hsps-mediated neuroprotection in diabetic nerve. Solid lines indicate stimulated pathways; dashed lines indicate inhibited pathways.

A study in *ex vivo* rat heart also found HSP90 was necessary for targeted mitochondrial import of PKCε and was critical for cardioprotection from ischemia/reperfusion injury (Budas *et al.*, 2010). It is also worth-mentioning that the expression of HSP60 and Grp75, two mitochondria-localized chaperones involved in organelle proteostasis, were decreased in myocardial mitochondria from STZ-rats (Turko and Murad, 2003). Decreased expression of Grp75 correlated with a decrease in the rate of protein import in interfibrillar mitochondria isolated from diabetic heart (Baseler et al., 2011). Since Grp75 is a key component of the

molecular motor complex involved in mitochondrial protein import, these data suggest that increasing HSP70 paralogs may have beneficial effects on mitochondrial dysfunction in DPN. Along this line, we have observed that KU-32 can translationally induce Grp75 in hyperglycemically stressed sensory neurons and are examining if this may contribute to improving mitochondrial function (unpublished observation). Recent work has also shown that genetic over-expression of Grp75 can increase Mn superoxide dismutase (MnSOD) expression, reduce markers of oxidative stress and decrease cell death induced by ischemia/reperfusion (Williamson *et al.*, 2008; Xu *et al.*, 2009). Since increased expression of MnSOD correlated with improved mitochondrial respiratory function in sensory neurons of diabetic mice treated with insulin (Akude et al 2011, Zherebitskaya etal., 2009), these data provide a potential mechanism by which increasing Grp75 may decrease oxidative stress and improve mitochondrial function in diabetic neurons. Interestingly, our unpublished preliminary data indicates that KU-32 also increased the translation of MnSOD and its expression in mitochondria of hyperglycemically stressed sensory neurons (unpublished data). Similarly, increasing expression of HSP60 with heat stress in myocardium was associated with enhanced mitochondrial complex activity which was believed to partially confer the protection against subsequent ischemia/reperfusion injury (Sammut *et al.*, 2001). Thus, increasing the expression of mitochondrial chaperones might offer a defense against mitochondrial dysfunction in diabetic neurons (Fig.2.).

2.4.3 Myelin protection

Segmental demyelination is a well characterized outcome of severe DPN in humans (Mizisin and Powell, 2003). However, it is difficult to assess the efficacy of increasing HSP70 in ameliorating diabetes-induced myelin degeneration since most diabetic rodent models do not recapitulate the observed myelinopathy in humans. Nonetheless, the ability of KU-32 to improve mechanical sensitivity in diabetic mice suggests that modulating chaperones can improve the function of myelinated fiber subtypes. Along this line, HSP70 is expressed in SCs of sciatic nerve but did not have a strong expression in axons (Fig.3.).

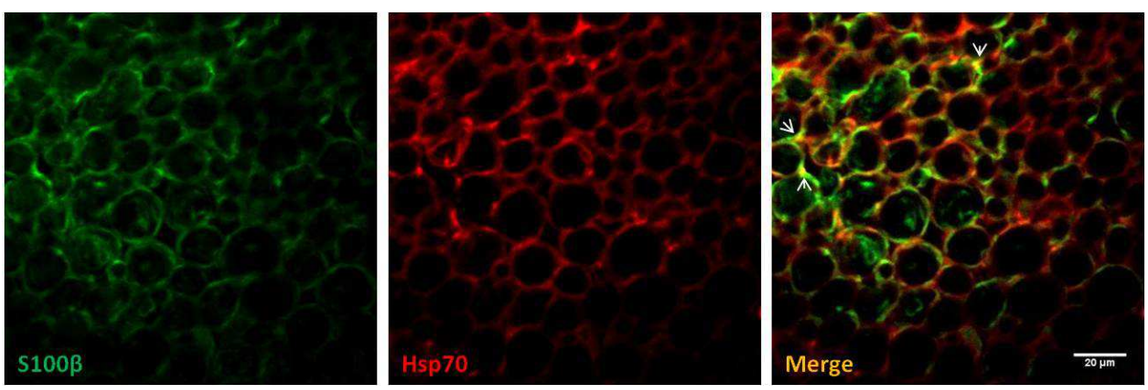

Fig. 3. Co-localization of HSP70 and SC marker in sciatic nerve. Sciatic nerves were removed from ~1 month-old C57Bl mice, fixed using 4% paraformaldehyde and cryoprotected using 30% sucrose before being embedded with tissue tek for freeze-sectioning (cross-section [10-micron]). Sections were then stained for HSP70 (red) and SC marker S100β (green). Arrowheads provide examples of co-localization of protein expression.

To more clearly localize the induction of HSP70 in peripheral nerve in response to KU-32 treatment, we are using transgenic mice that express green fluorescent protein under the control of the HSP70 promoter. However, our in vitro results using myelinated Schwann cell (SC)-sensory neuron co-cultures demonstrate that KU-32 pre-treatment effectively inhibits neuregulin-induced demyelination (Urban *et al.*, 2010). Similar to the result obtained in diabetic mice, HSP70 was required for this neuroprotection since KU-32 was unable to prevent neuregulin-induced demyelination in co-cultures prepared form HSP70 knockout mice. Although the mechanism by which HSP70 may prevent demyelination is unclear, induction of HSP70 in SCs by a small molecule HSP90 inhibitor significantly improved the myelination of DRG explants from a mouse model of hereditary demyelinating neuropathy, possibly via preventing the pathological misfolding and aggregation of myelin protein PMP22 (Rangaraju *et al.*, 2008).

2.4.4 Na^+/K^+-ATPase stabilization

Impaired Na^+/K^+-ATPase activity has been a frequent finding in nerves of diabetic humans and animals and strongly correlates with defects in NCV (Coste *et al.*, 1999; Greene *et al.*, 1987; Raccah *et al.*, 1994; Scarpini *et al.*, 1993). HSP70 directly interacts with the Na^+/K^+-ATPase and stabilizes its cytoskeletal anchorage in renal outer medulla (Ruete *et al.*, 2008) and epithelial cells (Riordan *et al.*, 2005) after ATP depletion. Whether this interaction may represent a fundamental mechanism underlying cellular protection is unknown, but warrants investigating if stabilization of Na^+/K^+-ATPase by HSP70 contributes to restoring NCV in diabetic nerves.

2.4.5 Protein chaperoning

Lastly, although protein misfolding or aggregation is not a primary pathological factor in DPN, excessive ROS production and protein glycation would unavoidably destabilize and damage cellular proteins and lead to their functional disruption and aggregation (Fig.2.). Indeed, deposition of advanced glycation end-products have been detected at the submicroscopic level as irregular aggregates in the cytoplasm of endothelial cells, pericytes, axoplasm and SCs of both myelinated and unmyelinated fibers in sural nerve biopsies of diabetic patients (Sugimoto *et al.*, 1997). Glycation of myelin also occurs and has been linked to abnormal NCV (Vlassara *et al.*, 1981). Hence, the chaperoning function of HSPs in promoting the health of diabetic nerve should not be overlooked.

3. Concluding remarks

With the climbing global prevalence of DPN and relatively scarcity of effective therapeutic strategies, novel approaches are needed to improve its medical management. The promising effects demonstrated by HSP70 in improving insulin resistance and DPN offers the exciting potential that induction of HSPs may be effective in improving both glycemic control and indirectly delaying the development of complications such as DPN in type 2 diabetics. However, the ability of HSP70 induction to ameliorate clinically relevant indices of DPN in the absence of improving metabolic control in models of type 1 DM also suggests that chaperones can directly affect neurons and SCs and retard disease progression. Given the decades long natural history of DPN, it is possible that, coupled with good glycemic control, combining inhibitors that target specific pathogenetic pathways with small molecule

inducers of the inherently cytoprotective biology associated with molecular chaperones may provide a powerful approach to decrease neuronal glucotoxicity and increase tolerance to recurring hyperglycemic stress.

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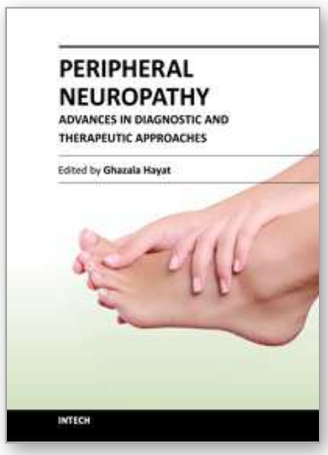

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Over the last two decades we have seen extensive progress within the practice of neurology. We have refined our understanding of the etiology and pathogenesis for both peripheral and central nervous system diseases, and developed new therapeutic approaches towards these diseases. Peripheral neuropathy is a common disorder seen by many specialists and can pose a diagnostic dilemma. Many etiologies, including drugs that are used to treat other diseases, can cause peripheral neuropathy. However, the most common cause is Diabetes Mellitus, a disease all physicians encounter. Disability due to peripheral neuropathy can be severe, as the patients suffer from symptoms daily. This book addresses the advances in the diagnosis and therapies of peripheral neuropathy over the last decade. The basics of different peripheral neuropathies is briefly discussed, however, the book focuses on topics that address new approaches to peripheral neuropathies.

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